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(54) PREPARATION PHARMACEUTIQUE CONTENANT DIVERS FACTEURS TRIBUTAIRES DE LA VITAMINE K  
 (54) PHARMACEUTICAL SUBSTANCE CONTAINING VARIOUS VITAMIN K-DEPENDENT FACTORS

(57)

The invention relates to a pharmaceutical substance separated from a prothrombin complex, containing at least two different blood factors which are highly purified and vitamin k-dependent.



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(54) **PREPARATION PHARMACEUTIQUE CONTENANT DIVERS  
FACTEURS TRIBUTAIRES DE LA VITAMINE K**  
(54) **PHARMACEUTICAL SUBSTANCE CONTAINING VARIOUS  
VITAMIN K-DEPENDENT FACTORS**

(57) La présente invention concerne une préparation pharmaceutique séparée d'un complexe de prothrombine, contenant au moins deux facteurs sanguins individuels, hautement purifiés et tributaires de la vitamine K.

(57) The invention relates to a pharmaceutical substance separated from a prothrombin complex, containing at least two different blood factors which are highly purified and vitamin k-dependent.



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A Pharmaceutical Preparation Comprising  
Vitamin K-Dependent Single Factors

A b s t r a c t :

A pharmaceutical separated prothrombin complex preparation is described which comprises at least two highly purified, vitamin K-dependent single blood factors.

**A Pharmaceutical Preparation Comprising  
Vitamin K-Dependent Single Factors**

The invention relates to a pharmaceutical preparation comprising vitamin K-dependent single factors.

Vitamin K-dependent proteins are characterized in that they essentially require vitamin K for their biosynthesis. Thus, e.g., prothrombin (factor II) formed under the influence of vitamin K antagonists, in contrast to normal prothrombin can not bind  $\text{Ca}^{2+}$ . Normal prothrombin contains  $\gamma$ -carboxyglutamate at the N-terminal end, i.e. a second carboxyl group at the glutamate residue. For, in the course of biogenesis of functional prothrombin, the first ten glutamate residues in the amino-terminal region of the protein are carboxylated to  $\gamma$ -carboxyglutamate by a vitamin K-dependent enzyme system. This  $\gamma$ -carboxyglutamate group is a very strong chelating agent for calcium ions. Via these bound  $\text{Ca}^{2+}$  ions, prothrombin is bound on phospholipid membranes which are derived from cell membranes, e.g. from blood platelets, to thus obtain the correct topology for initiation of blood coagulation.

It is not only prothrombin which has  $\gamma$ -carboxyglutamate residues, but also the coagulation factors VII, IX and X are carboxylated on specific glutamate

residues to thus form a high affinity relative to calcium ions. Yet also further proteins involved in the coagulation cascade, such as protein C, protein S and protein Z, require vitamin K for their biosynthesis.

Vitamin K-dependent single factors, in particular factors II, VII, IX and X, have similar physical-chemical properties, such as, e.g., similar mol weights, pIs, electrophoretic mobility, etc. and therefore as a rule are recovered together as prothrombin complex (other designation: factor IX complex or PPSB-complex). On account of the similar protein characteristic, it is difficult to prepare the factors individually. The production of prothrombin complex preparations with a simultaneous isolation of all the factors contained therefore has always been preferred in the prior art over the production of blood factor concentrates when producing pharmaceutical preparations (cf. Brummelhuis in: Methods of Plasma Protein Fractionation, ed. Curling, 1980, Academic Press, pp. 117-128). Yet it has also been shown that the prothrombin complex factors, on account of their different stabilities or half-lives, respectively, can never be obtained in a physiological ratio (always 1 U of the protein) (cf. Müller et al., Krankenhauspharmazie 13 (11), (1992), 528-531; Köhler et al., Thrombosis Research 60 (1990), pp. 63-70).

In EP-A 0 700 684, a prothrombin complex

concentrate together with at least one further blood-coagulation promoting component as an antidote for blood anticoagulants has been described.

There is the risk that prothrombin complex concentrates contain activated coagulation factors because they have been purified from complex protein mixtures, which activated coagulation factors in most instances are serine proteases. Yet particularly patients should not be coagulated, since the formation of thromboses may be or is, respectively, fatal. Even if only traces of these activated coagulation factors, in particular thrombin, are present in such a preparation, proteolytic inactivation of single factors will occur which, depending on the individual stability of the single factors, may have severe consequences. On the whole, thus, prothrombin complex concentrates tend to instabilities and are not suitable for an extended storage. These degradation reactions, in particular due to thrombin, occur even in the solid state, which means that also drying or lyophilisation, respectively, of the preparations will not result in a reliable storage stability.

Moreover, prothrombin complex concentrates are extremely unflexible as regards the relative ratios of the individual factors contained. There are hardly any possibilities to influence these relative ratios in the course of purifying the prothrombin complex, which is

particularly disadvantageous if a prothrombin complex in which certain factors are enriched is desired.

Thus, it is an object of the present invention to provide pharmaceutical combination preparations of vitamin K-dependent proteins which are, or can be, respectively, precisely defined as regards their composition, which have a high stability, in particular during extended storage, and which are highly flexible as regards variation of their composition.

According to the invention, this object is achieved by a pharmaceutical separated prothrombin complex preparation comprising at least two chromatographically purified vitamin K-dependent single factors as active substances. Above all, the preparation according to the invention shall contain highly purified factor IX in combination with at least one further highly purified vitamin K-dependent single factor.

In contrast to the prior art in which the prothrombin complex has always been purified as a complex and has not been prepared by combination of single factors and, moreover, even at its best is present only up to an intermediary, i.e. moderate, purity, with the present invention a preparation is provided which contains the individual blood factors in highly purified form, which are freed from interfering contaminations, in particular of a thrombin activity. In particular, the single factors to be combined

according to the invention are purified by chromatographic purification methods, such as ion exchange chromatography, hydrophobic chromatography, affinity chromatography and/or molecular exclusion chromatography, from plasma or recombinant cells. In this manner, specific activities of at least 50% of the theoretical purity, preferably at least 70%, in particular at least 90%, up to the theoretical purity can be attained for most vitamin K-dependent single factors in each case. Accordingly, it is also preferred to use factors which are substantially free ( $\leq 5\%$ ) of denaturing products.

In the pharmaceutical production of blood coagulation protein preparations, as a rule it is differentiated between three different degrees of purity: low, intermediary, and high.

Table 1

	<u>In vivo Half-Life</u>	<u>Theoretical Purity</u>
Factor II	60 h	0.1 U/mg
Factor VII	2 - 2.5 h	2000 (1667 - 2500)
Factor IX	18 - 24 h	250 (200 - 333)
Factor X	40 h	118 (100 - 143)

In particular when determining the factor II activity, the results repeatedly are falsified because

of the presence of traces of factor IIa, since even traces of factor IIa will interfere with the concentration determination of factor II such that values even by a multiple higher than the theoretical purity can be determined. With factor VII, the values therefore are somewhat lower relative to the other factors, since factor VII is a very labile protein which is extremely rapidly converted to factor VIIa. Therefore, even a factor VII preparation which has more than 10% of the theoretical purity is considered as highly purified.

Since the preparation according to the invention is composed of single factor preparations which are strictly defined particularly as regards their activities and their degrees of purity, respectively, also the ratio of the single factors to each other can be optimally adjusted. Thus, also problems occurring in the prior art in the course of further processing of the total protein complex concentrates, e.g. by activity losses during virus inactivation, but also by processes which occur in the course of the purification procedure, can be avoided from the very beginning, since after combining the highly purified single factors to the preparation according to the invention, preferably no further processing steps will be carried out.

This means that the preparation according to the

invention on the whole has the advantage of being standardizable. In this manner it is ensured that the respective individual factors in the added concentration are contained in the preparation +/- 10% deviation.

According to the invention, preferred single factors are selected from the group consisting of factor II, factor VII, factor IX, factor X, protein C, protein S and protein Z. Preferred production methods for highly purified preparations of these proteins can be found e.g. in EP 0 796 623 (factors II and X), A 594/97 (factor VII), EP 0 496 725 (factor IX), EP 0 533 209 (protein C) and EP 0 406 216 (protein S).

In the preparation according to the invention, preferably at least the factor II, VII, IX and X, starting from highly purified single factors, are combined, wherein, optionally, also the highly purified single factors protein C, protein S and/or protein Z are admixed, so as to be able to provide a prothrombin complex which is as physiological as possible, i.e. a prothrombin complex whose composition corresponds to the physiological one - yet without the interfering accompanying proteins which are included during the purification of the prothrombin complex from plasma, such as, e.g., thrombin.

The single factors may be purified from plasma, in particular human plasma, or be prepared by recombinant

technology. Since in the preparation by recombinant DNA technology a separation of the structurally and physically-chemically very similar factors is not necessary, the preparation according to the invention preferably is combined of highly purified recombinant single factors. The factor may also be transgenically prepared; it may be a derivative, in particular a peptide, and/or a fragment.

The vitamin K-dependent single factors, factor II, factor VII, factor IX, factor X, protein S and C, have been cloned and sequenced, and their production has, e.g., been described in Falkner et al., Thrombosis and Haemostasis 68 (2) (1992), pp. 119 to 124, for vitamin K-dependent proteins.

According to the invention, the relative ratios of the highly purified single factors are easily adjustable in any desired relation to each other within the preparation, e.g. in that these ratios correspond to the natural ratios in blood, i.e. in that approximately per unit of the one factor, one unit each of the other factor is present. A preferred preparation according to the present invention therefore contains the highly purified single factors, factor II, factor VII, factor IX and factor X at relative ratios, based on international units, of (0.5 to 2) : (0.5 to 2) : (0.5 to 2) : (0.5 to 2). If also protein C, protein S and/or protein Z are present in the preparation, also

these single factors preferably have relative ratios of from 0.5 to 2.

On the other hand, it is also possible according to the invention to adjust the individual factors, due to their relative stabilities, in particular at the ratios of their relative half-lives, i.e. that more is provided of a less stable factor, and, correspondingly, less of the stable factor. In this instance, also the intended period of time of application or action, respectively, may additionally be taken into consideration, i.e. the longer this time period, the higher also the relative ratios have to be considered.

Also recombinant proteins of, e.g., changed half-lives, may then be standardized accordingly. Also further factors, such as, e.g., the in vivo recovery, may be included in a standardization of the factors.

A preparation which is preferred in this respect therefore comprises the single factors, factor II, factor VII, factor IX and factor X at relative ratios, based on international units, of (0.5 to 2) : (5 to 35) : (0.5 to 7) : (0.5 to 5), since the half-lives of prothrombin are 60 hours, of factor VII 2 hours, of factor IX 20 hours and of factor X 40 hours. A preferred preparation with these factors therefore comprises the single factors, factor II, factor VII, factor IX, factor X, protein C and protein S at relative ratios, based on international units, of (0.5

to 2) : (5 to 35) : (0.5 to 7) : (0.5 to 5) : (1 to 15)  
: (1 to 15).

Since factor II by far is the most stable one of these factors and, moreover, also in its activated form thrombin carries the highest stability risk, a preparation which does not contain prothrombin is preferred. Factor VII mostly is considered as rather unstable, and therefore in the preparation according to the invention it is preferably provided to an increased extent, e.g. in 10-fold concentration (based on international units). A particularly preferred preparation thus contains single factor VII and single factor II at a ratio of greater 10 : 1.

With the present preparation, preferably a prothrombin complex or a partial prothrombin complex is provided from highly purified single factors. In any event, it is preferred that the single factors in the preparation do not form a complex. This may be shown, e.g., by analytical ion exchange chromatography on Q-Sepharose (Pharmacia), wherein the single factors during elution with a salt gradient can be discretely eluted. In contrast thereto are the complexes as they occur in prothrombinase or in pro-prothrombinase.

Prothrombinase is an enzyme-substrate complex which forms on a phospholipid surface and enables the activation of prothrombin. Prothrombinase by definition consists of factor II (prothrombin), activated factor X

(factor Xa) cofactors V and/or Va, respectively, phospholipids and calcium ions. *In vivo*, these factors are present as a transient complex for the activation of prothrombin and the formation of thrombin. A corresponding pro-prothrombinase is defined as a complex of factors which are present at least partially modified or activated, respectively, for the formation of a prothrombinase. Pro-prothrombinase therefore is to be understood as a precursor of prothrombinase and as a complex in which one or more components are present in their precursors, as zymogens, or as proforms and which is formed on the basis of affinities of the components to each other.

For stability reasons, it is advantageous to avoid any presence of activated coagulation factors in the preparation according to the invention. A preferred embodiment therefore is characterized in that the preparation does not comprise any activated coagulation factors, in particular does not comprise any factor IIIa, IXa, Xa and, optionally, VIIa.

Preferred preparations according to the invention comprise less than 0.1 U of factor VIII:C or factor VII:Ag/mg of protein and/or less than 0.1 U of factor IIa/unit of prothrombin and/or less than 0.1 U of factor Xa/unit of factor X.

To maintain the excellent stability of the preparation according to the invention as long as

possible, it is advantageous to provide the preparation according to the invention in lyophilized form. In this manner it is possible to store the preparation according to the invention for an almost unlimited period of time and nevertheless reconstitute it as a lyophilisate within a short period of time to a ready-to-use solution.

According to a further preferred embodiment, the preparation according to the invention further comprises magnesium ions. These ions act competitively to calcium ions and can displace the calcium ions primarily in the complete or partial prothrombin complex. In this manner, a premature thrombin formation in a solution of the preparation according to the invention is prevented to an even higher extent and the latter thus is stabilized so much that even in an aqueous solution it will remain stable for many hours.

It has been shown that the pharmaceutical preparation according to the invention can even be provided as a stable infusion solution, primarily if it is ensured that it does not contain any free calcium ions. The content of free calcium ions can easily be determined by the known ion titration or by other analytical methods. To complex the calcium ions, e.g. a pharmaceutically acceptable chelating agent, preferably EDTA, and related substances, such as citrate, are suitable.

Preferably, the preparation according to the invention furthermore comprises antithrombin III in those amounts in which hitherto it has been used in stabilizing manner in prothrombin complex concentrates, optionally together with heparin. Although this measure does not seem absolutely necessary because of the high degree of purity of the single factors, it may be considered advantageous for pharmaceutical reasons or also for requirements of pharmacopoeias or other rules with a view to the prothrombin complex concentrates of the prior art.

In another preferred embodiment, the preparation therefore is free of albumin and/or stabilizers, such as in particular antithrombin III and/or heparin. In particular, the preparation according to the invention is also free from phospholipids.

According to a preferred embodiment, the pharmaceutical preparation according to the invention is freed from infectious viruses or other infectious agents as a consequence of a treatment for virus inactivation. This treatment for virus inactivation preferably is ensured by two independent virus inactivation or virus depletion methods.

Preferably, this inactivation treatment is ensured by a tenside and/or heat treatment, e.g. by a heat treatment in the solid state, in particular a vapor treatment according to EP-0 159 311, EP-0 519 901 or

EP-0 674 531.

Further treatments for virus inactivation also comprise the treatment with chemical or chemical/physical methods, e.g. with chaotropic substances according to WO 94/13329, DE 44 34 538 or EP-0 131 740 (solvent) or photoinactivation.

Nanofiltration or the antibody-intensified nanofiltration (WO 9740861) also constitute a preferred method for virus depletion within the scope of the present invention.

Preferably, the preparation according to the invention further comprises pharmaceutically acceptable buffer substances or stabilizers, e.g. the substances already provided for prothrombin complex concentrates in the pharmacopoeias.

A particularly preferred variant for ensuring the freedom from viruses of the product according to the invention consists in that it is composed of highly purified vitamin K-dependent single factors which in turn have already been virus-inactivated and optionally already have been freed from denaturing products being formed and from stabilizers, so that they can be present in virus-inactivated and nevertheless precisely defined form as regards their activities.

Since above all in thermal virus inactivation methods, partial inactivation processes of the prothrombin factors may occur which, depending on the

stability of the single factor, may lead to different yields and specific activities after the thermal treatment of prothrombin complex preparations. One embodiment of the method according to the invention also allows for adjusting the ratios in the mixture of single factors such that even after a thermal treatment, the factors will be present at the desired ratios to each other. For instance, if it is known that the thermal treatment of prothrombin does not result in a loss of activity, whereas the factors, factor X and factor IX also present in the combination preparation will be inactivated by 20% each, a mixed, thermally virus-inactivated "complex" can be adjusted by combining the factors II, IX and X at activity ratios of 1 : 1.25 : 1.25. After this adjustment of the ratios, virus inactivation may then be carried out, and from this a preparation will directly result which comprises these factors at the ratio of 1 : 1 : 1.

Preferably, factor X is used in its  $\alpha$ -form and/or  $\beta$ -form.

A subject matter of the present invention is also a diagnostic preparation which according to the invention, is composed of the highly purified vitamin K-dependent single factors. Also for diagnosis, the advantages of definition, variability of concentration ratios and stability are of particular advantage.

The pharmaceutical preparation according to the

invention may, of course, be used for all other previous indications of the prothrombin complex.

Thus, a subject matter of the present invention is also the use of the preparation according to the invention for producing a preparation for the treatment of acquired or inherited blood coagulation disorders, for the treatment of severe hemorrhages, for the prophylaxis of hemorrhages, in particular if inherited blood coagulation disorders are present, for substitution therapy and for the treatment of hemophilia B.

Also for liver dysfunctions, the preparation according to the invention proves to be indicated.

When administered to a patient, the administration regimen has to be taken as a basis for dosage, wherein, however, the more precise definition of the preparations of the invention is advantageous.

The present invention will be explained in more detail by way of the following examples to which, however, it shall not be restricted.

#### **E x a m p l e s :**

##### **E x a m p l e 1 : Production of the single factor preparations**

###### **1.1 Production of single factor preparations of plasmatic factor X and plasmatic factor II:**

A lyophilized prothrombin complex factor preparation which contained factors II, IX, X as well

as protein C and protein S was prepared according to the method of Brummelhuis, H.G.J., Preparation of the Prothrombin complex. In: Methods of Plasma Protein Fractionation, Curling, J.M. ed., 117-128, Academic Press, New York, (1980), and heat-treated for virus inactivation according to EP 159 311. Accordingly, the lyophilisate (1,000 U of factor X/g, 1,200 U of factor II/g) was dissolved in distilled water so that the latter contained 50,000 U of factor X/l, and adjusted to pH 7.0. After addition of 12% (v/v) Tween® 80, it was stirred for 1 hour at room temperature. Subsequently, it was diluted 1:5 with a 20 mM Tris-HCl buffer, pH 7.0, and the prothrombin complex protein fraction was adsorbed on calcium phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ ) at a concentration of 30 g of  $\text{Ca}_3(\text{PO}_4)_2$  per l of prothrombin complex solution by stirring for 1 h at room temperature. Subsequently, the solid phase was separated by centrifugation, 20 min at 5,000 rpm, and the precipitate was washed twice with 20 mM Tris-HCl buffer, pH 7.0, containing 10% ammonium sulfate, by resuspension and renewed centrifugation. A third washing was carried out in an analogous manner with 20 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl. Elution of the prothrombin complex fraction was effected with 1 M sodium phosphate solution, pH 7.0, wherein 25 ml of this solution per g of calcium phosphate were stirred for 1 hour at room temperature.

and subsequently the remaining precipitate was separated by centrifugation as above. Subsequently, the supernatant was subjected to an ammonium sulfate precipitation with 366 g of ammonium sulfate per l for 15 hours at 4°C under stirring. The precipitate containing the prothrombin complex fraction was separated by centrifugation as above. The precipitate was taken up in a 25 mM trisodium citrate dihydrate buffer containing 100 mM NaCl, 1 mM benzamidine hydrochloride, pH 6.0, and rebuffered on a column filled with Sephadex® G-25 at 4°C with a linear flow of 1 cm/min against 25 mM trisodium citrate dihydrate buffer containing 100 mM NaCl and 1 mM benzamidine hydrochloride, pH 6.0, so as to separate the ammonium sulfate. In doing so, the UV absorption at 280 nm and the electric conductivity were measured in the eluate flow. The protein-containing fractions were combined and subsequently subjected to an ion exchange chromatography over DEAE-Sephadex FF® (Pharmacia). The fractions were applied on a column (inner diameter : gel bed height = 1 : 1.3) with a gel volume of 8.2 l, 0.55 g of protein/l of gel, at a linear flow of 0.36 cm/min. Chromatography was carried out at 22°C. Before the proteins were applied, the column had been equilibrated with a 25 mM trisodium citrate dihydrate buffer, containing 100 mM NaCl, 1 mM benzamidine hydrochloride, pH 6.0. Elution of the protein fractions

was carried out in several steps with a buffer 1 (25 mM trisodium citrate dihydrate, 1 mM benzamidine hydrochloride, 245 mM NaCl, pH 6.0), buffer 2 (25 mM trisodium citrate dihydrate, 1 mM benzamidine hydrochloride, 270 mM NaCl, pH 6.0) and a buffer 3 (25 mM trisodium citrate dihydrate, 1 mM benzamidine hydrochloride, 400 mM NaCl, pH 6.0). Elution with buffer 1 was carried out with 2.4 column volumes, and in doing so, inert protein was separated. Elution was carried out with 5.6 column volumes in buffer 2, and here the fractions were collected which were analyzed for their contents of factor II, factor X, protein C and factor IX. The factor X-containing fractions which were free from factors II, IX and protein C were combined. This highly purified factor X preparation had a specific activity of 60 U/mg of protein.

By elution with buffer 3 (1.9 column volumes), factor II was desorbed, wherein, again, the fractions were collected and assayed for their contents of factor X, factor IX and factor II. The factor II-containing fractions were pooled. Both factor II and also the factor X-containing pool optionally could be subjected to an additional treatment for inactivation of pathogenic impurities by the addition of 1 M KSCN and incubation at 22°C for several hours.

The thus recovered factor II pool was adjusted to 1.8 M NaCl by the addition of sodium chloride, and the

pH was corrected to pH 7.0. Subsequently, this solution was adsorbed on a gel, Phenylsepharose High Performance® (Pharmacia), by hydrophobic interaction, whereby 3 g of protein/l of gel being bound. In a column with a ratio of inner diameter : gel bed height = 1 : 1.9, the protein fraction was adsorbed at a linear flow of 0.25 cm/min, and subsequently was freed from inert protein by washing with a buffer (25 mM Tris-HCl, 3 M NaCl, pH 7.4). By gradient elution with 11.5 column volumes of 3 M - 0.9 M NaCl with simultaneous collection of fractions, factor II was eluted from the column. Those fractions which contained factor II activity were pooled and yet were free from factors X and IX. Subsequently, the collected factor II-fractions were concentrated 10 times by ultra/diafiltration via an ultrafiltration membrane having a cut-off of 30 kD, and rebuffered against a buffer containing 4 g of trisodium citrate dihydrate/l, 8 g of NaCl/l, pH 7.0. A thus prepared factor II-preparation had a specific activity of 6.9 U/mg of protein. Determination of the factor II activity was carried out with the 1 step method, based on the thromboplastin time, by using a factor II deficiency plasma against the International Factor II Standard, employing the reagent combination from BAXTER, Vienna. In the coagulation analyses, other coagulation factors were detectable in traces or were not detectable any

longer (factor VII < 0.00002 U/U factor II, factor IX 0.0002 U/U factor II, factor X 0.004 U/U factor II, protein C 0.003 U/U factor II, and factor VIII < 0.0002 U/U factor II).

As an alternative production method for a highly purified factor II, also a method was used in which first factor IX was separated from a lyophilized prothrombin complex factor preparation by hydrophobic chromatography, subsequently factor II was isolated, and the latter was highly purified by chromatography on hydroxyl apatite.

The prothrombin complex factor preparation was dissolved as described above and incubated with a detergent for 1 h at room temperature. Subsequently, a factor II, IX and X-containing fraction was isolated by ion exchange chromatography on DEAE Sepharose FF® (Pharmacia). From this, subsequently the factor IX-containing fraction was removed by interaction with Butyl-Toyopearl® (Toso Haas). The adsorption supernatant subsequently was purified on Phenyl-Sepharose High Performance® (Pharmacia) by a further hydrophobic interaction chromatography, wherein approximately 4 g of protein/l of gel could be adsorbed. In a column having a ratio of inner diameter : gel bed height = 1 : 1.9, the protein fraction was adsorbed at a linear flow of 0.25 cm/min, subsequently the inert protein was removed by washing with 20 mM

Tris-HCl, 3 M NaCl, pH 7.4, and finally the factor II-containing fraction was isolated by step-wise elution, which fraction desorbed from the gel at 1.9 M NaCl at decreasing conductivity. The factor II-containing fraction then was directly adsorbed on Ceramik-Hydroxylapatit® (BioRad). This was carried out on a column with a ratio of inner diameter : gel bed height = 1 : 4.8. Elution was effected at a linear flow of 3 cm/min. By elution with a salt gradient, factor II could be desorbed from the column. The factor II-containing fractions were collected and concentrated by ultra/diafiltration via polysulfone membranes having a cut-off of 30 kD, until the factor II-concentration was 50-100 U/ml. A thus-prepared factor II preparation had a specific activity of at least 7 U/mg protein. Other coagulation factors, in particular factor IX and factor VIII, were merely detectable in traces or not detectable at all. By selecting a suitable diafiltration buffer, the factor II preparation was transferred into a pharmaceutically acceptable buffer (e.g. 4 g trisodium citrate dihydrate/l, 8 g NaCl/l, pH 7.0).

#### **1.2 Recovery of plasmatic factor VII:**

30 ml of fresh frozen human citrated plasma were thawed at 0 - +4°C, and the cryoprecipitate incurred was separated by centrifugation at +2°C. The "cryosupernatant" resulting therefrom was admixed with

2 IU of heparin/ml. Subsequently, the proteins of the prothrombin complex were adsorbed with DEAE-Sephadex® A-50 (Pharmacia) at a concentration of 0.5 mg/ml. The gel-protein complex was separated from the solution and washed respectively with buffer 1 (4 g/l Na<sub>3</sub>citrate·2H<sub>2</sub>O, 7 g/l NaCl, 9 g/l Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 500 IU heparin/l, pH 7.5) and subsequently with buffer 2 (4 g/l Na<sub>3</sub>citrate·2H<sub>2</sub>O/l, 7 g/l NaCl, 500 IU heparin/l, pH 7.5).

In this manner, the prothrombin complex fraction comprising the coagulation factors prothrombin, small amounts of factor VII, factor IX and factor X was separated. The main portion of coagulation factor VII remaining in the supernatant after adsorption on DEAE-Sephadex® A50 was then recovered by adsorption on aluminum hydroxide. Thereto, 10 ml of a 2% aluminum-hydrogel suspension were admixed per 1 l of supernatant after the prothrombin complex has been separated, and it was stirred at 4°C for 30 minutes. Subsequently, the aluminum hydroxide-protein complex was separated by means of centrifugation at 5000 rpm for 10 minutes at approximately 4°C in a Sorvall RC3B Rotor H6000A. The supernatant was discarded, and the precipitate was suspended with 3.5% of the volume of the prothrombin complex supernatant used for adsorption in a solution of 4 g Na<sub>3</sub>citrate·2H<sub>2</sub>O/l and 7 g NaCl/l, pH 7.5, and stirred for 30 minutes. In this manner the inert

protein was desorbed from aluminum hydroxide. Factor VII remaining on the aluminum hydroxide was pelletized by renewed centrifugation as described above. The supernatant was discarded, and the precipitate was used for further processing. For desorption of the protein fraction, the aluminum hydroxide factor VII complex was stirred for 30 minutes with 1 vol.-% of the prothrombin complex supernatant of an 0.3 mol/l phosphate buffer used for adsorption, pH 8.6 (53.4 g Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O/l were adjusted to pH 8.6 with a solution of 41.1 g NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O/l) containing 1% TWEEN®80. For pathogen inactivation, subsequently TWEEN®-80 detergent was admixed to a final concentration of 15%, followed by stirring for 1 h at 40°C.

After the solution had been cooled to 22°C, one aliquot of 20 ml was rebuffered by means of column chromatography via Sephadex® G-50 (Pharmacia) against a solution of 4 g Na<sub>3</sub>citrate.2H<sub>2</sub>O, pH 7.4, and diluted to the 10-fold with the same solution. Then the entire solution was applied onto a column which was packed with heparin sepharose CL6B (Pharmacia), having a diameter of 16 mm and a height of 10 cm. The flow rate was 1 ml/min. Subsequently, it was eluted with the 10-fold column volume with a salt gradient of from 0-1 M NaCl in a solution of 4 g Na<sub>3</sub>citrate.2H<sub>2</sub>O/l, pH 7.4. The protein fraction was quantitated by measuring UV absorption at 280 nm. Simultaneously, fractions were

collected, and factor VII was determined therein with a chromogenic factor VII assay (Immunochrom Faktor VII:10, BAXTER AG, Vienna). The fractions of the eluted protein which contained factor VII were collected and combined. The elution volume of the factor VII fraction was 25 ml. In the protein fraction, the protein content was determined according to the method of Bradford, M.M. (Anal. Biochem. 72: 248-254, 1976), and factor VII as described above. Likewise, factor VIIa was quantitated according to the method of US 5,472,850 (Ser. No. 683682). From this it could be calculated that the eluting factor VII fraction had a specific activity of 98 units/mg of protein, while the factor VIIa content was below one unit/ml. Accordingly, a highly purified factor VII preparation without any substantial activation of factor VII could be recovered. The yield at the chromatography was more than 50%.

### 1.3 Plasmatic factor IX:

150 ml of prothrombin complex were pre-purified by means of dextrane sulfate (Miletich et al., Analytical Biochemistry 105, 304 (1980)). 20 ml of eluate (912 I.U. of factor IX, 160 I.U. of factor X) were applied on a column with 20 ml of an agarose polymer with octyl groups (Octyl-Sepharose-CL-4B (Pharmacia, Sweden)) with a flow rate of 360 ml/h. The column previously had been equilibrated with 80 ml of buffer A. After washing of

the loaded gel with 120 ml of buffer A, the factor IX-containing fraction was eluted with 80 ml of a 250 mmol NaCl solution.

The yield of factor IX was approximately 54% of the starting activity. The specific activity was 186 I.U. of factor IX/mg of protein. Factor X was only present in traces any more.

#### 1.4 Recovery of plasmatic protein C

Highly pure protein C was recovered from a crude protein C fraction which was prepared from commercially obtainable prothrombin complex concentrate.

Purification was carried out by affinity chromatography by means of monoclonal antibodies. Monoclonal anti-protein C antibody was prepared as follows:

BALB/C mice were immunized by intraperitoneal injection with 100 µg of human protein C at two-week intervals. After six weeks, once again 50 µg of the human protein C were injected, and 3 days afterwards fusion was carried out. The myeloma cell line (P3-X-63-AG8-653,  $1.5 \times 10^7$  cells) was mixed with  $1.7 \times 10^8$  spleen cells from a mouse, and fusion was effected according to a modified Köhler & Milstein method by using PEG 1500 (Köhler G., Milstein C., Nature 256 (1975), 495-497).

Positive clones, tested by means of an ELISA, were subcloned twice. Ascites production was effected by injection of  $5 \times 10^6$  hybridoma cells per BALB/C mouse

two weeks after Pristan treatment.

The immunoglobulin was purified from ascites by ammonium sulfate precipitation and subsequent chromatography by means of QAE-Sephadex and, thereafter, chromatography on Sephadex G200. To reduce the risk of transmitting murine viruses, the antibody was additionally subjected to a virus inactivation step prior to immobilisation. The thus obtained monoclonal antibodies against protein C were coupled to CNBR-Sepharose 4B (Pharmacia). To purify the protein C by means of affinity chromatography, the following buffers were used:

as adsorption buffer: 20 mM Tris, 2 mM EDTA, 0.25 M NaCl and 5 mM benzamidine;

as washing buffer, 20 mM Tris, 1 M NaCl, 2 mM benzamidine, 2 mM EDTA was used; the pH was 7.4;

as elution buffer, 3 M NaSCN, 20 mM Tris, 1 M NaCl, 0.5 mM benzamidine, 2 mM EDTA was used.

A further suitable monoclonal antibody for purifying protein C has been described in US 5,336,610. This antibody binds to the activation peptide of protein C and thus is suitable to selectively purify the zymogen protein C from the activated form.

In detail: The prothrombin complex concentrate was dissolved in adsorption buffer, wherein approximately 10 g of the prothrombin complex concentrate were used for a 20 ml monoclonal antibody column. Subsequently,

the dissolved prothrombin complex concentrate was filtered, centrifuged for 15 min at 20,000 rpm, and sterile-filtered through a 0.8 µm filter. The sterile-filtered and dissolved prothrombin complex concentrate was applied to a column at a flow rate of 10 ml/h. Then the column was washed with a washing buffer so as to be free from protein, and finally the bound protein C was eluted with the elution buffer at a flow rate of 5 ml/h, and the fractions were collected. The eluted protein C was dialyzed against a buffer (0.2 M Tris, 0.15 M glycine, and 1 mM EDTA, pH 8.3). The protein C content was determined with respect to antigen by means of the method according to Laurell and with respect to activity after Protac activation.

The protein C eluate obtained was finished to a pharmaceutically applicable preparation in the following manner:

At first, the eluate was subjected to an ultrafiltration and a diafiltration step. For diafiltration, a buffer having a pH of 7.4 was used, which contained 150 mM NaCl and 15 mM trisodium citrate.2H<sub>2</sub>O. The filtrate obtained was freeze-dried and virus inactivated by a one-hour vapor treatment at 80°C ± 5°C and 1375 ± 35 mbar.

The lyophilized, virus-inactivated material then was dissolved in a sterile isotonic NaCl solution, and possibly present antibodies or serum amyloid P,

respectively, were removed by means of ion exchange chromatography on Q-Sepharose. The purified solution was concentrated by a further ultrafiltration and diafiltration step. Thereafter, 10 g albumin, 150 mM NaCl and 15 mM trisodium citrate were added. The pH of the solution was 7.5. Murine immunoglobulin as well as the factors II, VII, IX and X could not be detected. Subsequently, the solution was sterile-filtered, filled into containers and lyophilized. The specific activity was 14 units of protein C/mg. As the activity assay, an amidolytic assay was used wherein protein C was activated by means of Protac (Pentapharm).

#### 1.5 Purification of plasmatic protein S

Human protein S was prepared from factor IX concentrate (Prothrombin complex STIM-3, BAXTER AG, Vienna) by means of QAE-Sephadex and Blue Sepharose CL-6B chromatography (Pharmacia) in the following manner:

The lyophilized concentrate (100 g) was dissolved in 200 ml of sterile, ion-free water and dialyzed against a buffer consisting of 0.01 M 2-(N-morpholinoethane-sulfonic acid), pH 6.0; 0.18 M NaCl, 10 mM EDTA, 2 mM benzamidine-HCl and 0.02% NaN<sub>3</sub>, (starting buffer). Then the dialyzed material was applied to a QAE-Sephadex column (8 x 19 cm) and equilibrated with the buffer mentioned. As washing solution, 1.5 l of buffer (starting buffer) were used.

Protein S was eluted at 110 ml/h with a linear NaCl

gradient consisting of 1.2 l of starting buffer and 1.2 l of a further buffer which differs from the first buffer by the addition of 0.5 M NaCl. The protein S fractions were examined for protein S by means of Fast Flow SDS PAGE (Pharmacia) and antigen determination (Laurell). Protein S-containing fractions were pooled and finally dialyzed against a buffer. This buffer contained 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM benzamidine-HCl and 0.2% NaN<sub>3</sub>. After dialysis, the protein S pool was applied onto a Blue Sepharose column CL-6B (2.5 cm x 10.5 cm) and equilibrated with the starting buffer.

Washing was carried out with 500 ml of starting buffer at a flow rate of 15 ml/h. Thus, protein S could be eluted in the void volume, whereas prothrombin adsorbed to the column. Again, the protein S rich fractions were determined by means of SDS-PAGE Fast Flow System (Pharmacia) and according to Laurell (Scand. J. Clin. Invest. (Suppl.) 29 (1977) 21 (Suppl. 124)).

On a reduced SDS-PAGE, the thus prepared protein S had a morphology characteristic of protein S, namely two close bands (doublet) with a molecular weight of approximately 86,000 and 76,000, respectively. The protein concentration was determined spectrophotometrically by means of an extinction coefficient of 0.1 at 280 nm for human protein S and was confirmed by the

Method according to LOWRY (Lowry O. et al., Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265).

The thus prepared, pre-purified protein S was used for preparing sheep antiserum against protein S by carrying out four immunizing injections. 100 µg of protein S had been applied subcutaneously with Freund's adjuvant in the first two injections and incomplete adjuvant being employed in the following boosters. After further boosters, the antiserum was tested by means of double immunodiffusion and showed a precipitation with purified protein S and with normal plasma.

The IgG fraction from 450 ml antiserum was obtained by alcohol precipitation and subsequent adsorption on Sephadex A 50 in TRIS-HCl buffer, pH 6.8. From 450 ml of antiserum, 1.14 g of anti-protein S-IgG were present in the supernatant. The IgG fraction was coupled to 450 ml of Sepharose CL-4B, with 5.7 mg protein/ml Sepharose being used. The coupling efficiency was 76%. The anti-protein S column was equilibrated with glycine-HCl, pH 3, and adsorption buffer, pH 7.5.

The adsorption buffer was comprised of 20 mM TRIS, 2 mM EDTA, 0.25 M NaCl, 2 mM benzamidine, 0.02% Tween® 20 and 0.02% NaN<sub>3</sub>, pH 7.4. The washing buffer solution had the following contents: 20 mM TRIS, 2 mM EDTA, 1.0 M NaCl, 0.5 mM benzamidine, 0.01% Tween® 20;

0.02%  $\text{NaN}_3$ , pH 7.4.

The elution buffer had a composition like the washing buffer solution, except that 0.05% Tween® 20 and, additionally, 243.3 g of NaSCN, pH 7.4 (a 3 M rhodanid solution) were used.

The dialysis buffer solution contained 20 mM Tris, 0.15 mM glycine, 1 mM EDTA, 2 mM benzamidine, pH 8.3.

For further purification of the protein S fraction prepared from prothrombin complex concentrate, 100 g of the fraction were dissolved in 1 l of adsorption buffer and dialyzed over night against an adsorption buffer solution. After application of the sample onto the column, the column was washed with washing buffer, approximately 5 l, to be free from protein, subsequently elution was carried out with 3 M NaSCN in the elution buffer solution. The eluate was dialyzed immediately until SCN was below the detection limit; the eluate had a concentration of 500  $\mu\text{g}/\text{ml}$  protein S. It was free from C4-binding protein.

Monoclonal anti-protein S antibodies were prepared as follows:

BALB/C mice were immunized at two-week intervals by intraperitoneal injection of 100  $\mu\text{g}$  of the protein S produced. After six weeks, once again 50  $\mu\text{g}$  of the human protein S were injected, and 3 days afterwards fusion was carried out. The myeloma cell line (P3-X-63-AG8-653,  $1.5 \times 10^7$  cells) was mixed with  $1.7 \times 10^8$

spleen cells from a mouse, and fusion was effected according to a modified Köhler & Milstein method by using PEG 1500 (Köhler G., Milstein C., Nature 256 (1975), 495-497).

Positive clones, tested by means of an ELISA, were subcloned twice. Ascites production was effected by injection of  $5 \times 10^6$  hybridoma cells per BALB/C mouse two weeks after Pristan treatment.

The immunoglobulin was purified from ascites by ammonium sulfate precipitation and subsequent chromatography by means of QAE-Sephadex and, thereafter, chromatography on Sephadex G200.

The IgG fraction obtained from ascites and pre-purified on protein A-Sepharose was coupled to Sepharose CL-4B. The affinity chromatographic purification of protein S which had been recovered from the prothrombin complex concentrate was effected under the conditions described for polyclonal protein S antibodies. The concentration of the eluate on protein S was 600 µg/ml. It was free from C4-binding protein.

The protein S preparations highly purified according to the method of polyclonal or monoclonal affinity chromatography were subjected to an SDS-PAGE (gradient gel 8 to 12%), and were designated as more than 95% pure according to Coomassie staining (Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970),

680).

Subsequently, the eluates were subjected to an ultrafiltration and a diafiltration step. For diafiltration, a buffer having a pH of 7.4 was used which contained 150 mM NaCl and 15 mM trisodium citrate.2H<sub>2</sub>O. The filtrates obtained were freeze-dried and virus-inactivated by a one-hour vapor treatment at 80°C ± 5°C and 1375 ± 35 mbar (to remove possibly present viral contaminations of polyclonal or monoclonal antibody).

Then the lyophilized virus-inactivated material was dissolved in a sterile isotonic NaCl solution and possibly present antibodies and serum amyloid P, respectively, were removed by ion exchange chromatography on Q-Sepharose. The purified solution was concentrated by a further ultrafiltration and diafiltration step. Thereafter, 10 g of albumin, 150 mM NaCl and 15 mM trisodium citrate were added per liter to the solution obtained. The pH of the solution was 7.5. It contained 3000 µg/ml protein S. This content of protein S corresponds to a 500-fold enrichment as compared to plasma. Murine immunoglobulin as well as the factors II, VII, IX and X could not be detected. Subsequently, the solution was sterile-filtered, filled into containers and lyophilized.

**E x a m p l e 2 : Pharmaceutical preparation of highly purified vitamin K-dependent proteins:**

The highly purified single factors prothrombin, factor VII, factor IX, factor X, protein C and protein S were combined such that a solution containing 25,000 units/l of each of the single factors was formed in aqueous solution of 4 g Na<sub>3</sub>Citrate·2H<sub>2</sub>O and 8 g NaCl/l. The solution was sterile-filtered via a nylon filter having a retention rate of 0.2 µm and subsequently filled at 20 ml each in sterilized glass bottles having a filling volume of 50 ml. It was freeze-dried under sterile conditions, whereafter the bottles were closed under sterile conditions.

**E x a m p l e 3 : Pharmaceutical preparation of the prothrombin complex:**

As described in the previous example, a highly purified prothrombin complex comprising the factors prothrombin, factor VII, factor IX and factor X, yet without protein C and protein S, was prepared and filled into containers under sterile conditions. According to international recommendations, the solution was admixed with 10,000 international units of heparin/l before filling into containers.

**E x a m p l e 4 : Pharmaceutical preparation of vitamin K-dependent proteins for continuous substitution and maintaining of constant plasma levels:**

After substitution with a vitamin K-dependent protein concentrate in which plasma levels as constant as possible of the factors of the prothrombin complex

are to be attained, due to the different half-lives of the individual factors it is necessary to carry out the substitution rates of these proteins in a non-equivalent ratio. Accordingly, a pharmaceutical preparation as described above was produced which contained prothrombin, factor VII, factor IX, factor X, protein C and protein S at a ratio of 1:30:3:1.5:6:6.

**E x a m p l e 5 : Pharmaceutical preparation of a partial prothrombin complex:**

On account of the different *in vivo* half-lives of the factors of the prothrombin complex, in particular because of the long half-life of prothrombin of 2-5 days as compared to the short half-life of factor VII of 4-7 h, and of the factor IX of less than 24 h, in the continuous substitution with prothrombin complex preparations it happens that when adjusting a normal plasma concentration of 1 unit of factor VII/ml, already after 24 h the plasma concentration has decreased again to below the concentration of at least 20% required for a functioning haemostasis, whereas, e.g., the prothrombin plasma level still is approximately normal, i.e. is at 1 unit/ml. A repeated substitution with a prothrombin complex concentrate of the same composition as in the primary substitution will have the result that the prothrombin plasma concentration in the patient will be increased to above the normal range so as to attain once more equivalent

factor VII or factor IX plasma concentrations. Raising the plasma prothrombin concentration to above the standard range, however, means an increased risk of thrombosis for the patient treated, since Poort et al. (Blood 88:3698-3703, 1996) have found that a plasma level of prothrombin raised by as little as 30% involves a significantly increased risk of venous thromboses. This problem can be avoided by using combined prothrombin complex preparations for secondary substitutions.

Such a preparation is produced as follows: As described in the previous examples, a prothrombin complex concentrate comprising factor VII, IX and X at a ratio of 30:3:1.5, yet without prothrombin, is prepared and provided as a pharmaceutical formulation. The containers of this preparation intended for administration each contained 500 international units of the coagulation factors VII, IX and X in a dissolution volume of 10 ml after reconstitution of the lyophilized powder.

**E x a m p l e 6 : Quality check of the prothrombin complex preparations:**

The individual coagulation factors in the final containers of the prothrombin complex preparations were assayed for their content of prothrombin, factor VII, factor IX, factor X, protein C and protein S by means of 1-step coagulation tests according to standard

methods, by using deficient plasmas and coagulation reagents from BAXTER, Vienna.

Details to the methods used can be taken from the paper by Müller, H.G. and Bonik K. (Krankenhauspharmazie 13:528-531, 1992).

To determine the content of activated prothrombin complex factors, the chromogenic substrates S-2238, S-2222, S-2444, S-2366 and S-2251 (Chromogenix) were used. The determinations were carried out by using the buffer conditions indicated by the producer. With the chromogenic substrates, in each case the activated factors thrombin, factor VIIa, factor IXa, factor Xa, activated protein C which developed proteolytic activities against the peptide substrates used, could be determined. After evaluation of the photometric analysis of the turnover of the chromogenic substrate it could be determined that for all the substrates the content in the previously described preparation comprising vitamin K dependent proteins or selected factors of the prothrombin complex was less than 10 mU/ml in each case.

## C l a i m s :

1. A pharmaceutical, separated prothrombin complex preparation, characterized in that it comprises at least 2 chromatographically purified vitamin K-dependent single factors as the active substances.
2. A preparation according to claim 1, characterized in that the single factors are selected from the group of factor II, factor VII, factor IX, factor X, protein C, protein S and protein Z.
3. A preparation according to claim 1 or 2, characterized in that it comprises at least the factors II, VII, IX and X.
4. A preparation according to any one of claims 1 to 3, characterized in that it comprises the single factors protein C and protein S.
5. A preparation according to any one of claims 1 to 4, characterized in that at least one of the single factors is a recombinant factor, a transgenically prepared factor, a derivative, in particular a peptide, and/or a fragment.
6. A preparation according to any one of claims 1 to

5. characterized in that it comprises the single factors contained at a ratio which substantially corresponds to the ratio of these factors in blood.

7. A preparation according to any one of claims 1 to 6, characterized in that it comprises the single factors factor II, factor VII, factor IX and factor X at relative ratios, based on international units, of (0.5 to 2) : (0.5 to 2) : (0.5 to 2) : (0.5 to 2).

8. A preparation according to any one of claims 1 to 7, characterized in that it comprises the individual factors factor II, factor VII, factor IX, factor X, protein C and protein S at relative ratios, based on international units, of (0.5 to 2) : (0.5 to 2).

9. A preparation according to any one of claims 1 to 5, characterized in that it comprises the single factors contained at ratios which correspond to the relative half-lives of the single factors.

10. A preparation according to claim 9, characterized in that it comprises the single factors factor II, factor VII, factor IX and factor X at relative ratios, based on international units, of (0.5 to 2) : (5 to 35) : (0.5 to 7) : (0.5 to 5).

11. A preparation according to any one of claims 9 or 10, characterized in that it comprises the single factors factor II, factor VII, factor IX, factor X, protein C and protein S at relative ratios, based on international units, of (0.5 to 2) : (5 to 35) : (0.5 to 7) : (0.5 to 5) : (1 to 15) : (1 to 15).
12. A preparation according to claim 1, 2, 4, 5, 6 or 9, characterized in that it comprises factor VII and factor II at a ratio of greater than 10 : 1.
13. A preparation according to any one of claims 1 to 12, characterized in that the single factors in the preparation do not form a complex.
14. A preparation according to any one of claims 1 to 13, characterized in that it comprises a partial prothrombin complex.
15. A preparation according to any one of claims 1 to 14, characterized in that it does not contain an activated coagulation factor selected from IIa, IXa, Xa and, optionally, VIIa.
16. A preparation according to any one of claims 1 to 15, characterized in that it contains less than 0.1 U

of factor VIII:C or factor VIII:Ag/mg of protein.

17. A preparation according to any one of claims 1 to 16, characterized in that it contains less than 0.1 U of factor IIa/U of prothrombin.

18. A preparation according to any one of claims 1 to 17, characterized in that it contains less than 0.1 U of factor Xa/U of factor X.

19. A preparation according to any one of claims 1 to 18, characterized in that it is present in lyophilized form.

20. A preparation according to any one of claims 1 to 19, characterized in that it comprises magnesium ions.

21. A preparation according to any one of claims 1 to 20, characterized in that it does not contain free calcium ions.

22. A preparation according to claim 21, characterized in that it comprises a chelating agent for complexing free calcium ions.

23. A preparation according to any one of claims 1 to 22, characterized in that it further comprises

antithrombin III in stabilizing amounts, optionally together with heparin.

24. A preparation according to any one of claims 1 to 23, characterized in that it is free from infectious viruses on account of a virus inactivation or virus depletion treatment.

25. A preparation according to claim 24, characterized in that its freedom from infectious viruses is ensured by two independent virus inactivation or virus depletion methods.

26. A preparation according to any one of claims 1 to 25, characterized in that it further comprises pharmaceutically acceptable buffer substances or stabilizers.

27. A preparation according to any one of claims 1 to 26, characterized in that it comprises highly purified vitamin K-dependent single factors which are virus-inactivated.

28. A preparation according to any one of claims 1 to 27, characterized in that it comprises the single factor X in its  $\alpha$ -form and/or in its  $\beta$ -form.

29. A diagnostical preparation, comprising a preparation according to any one of claims 1 to 28.

30. The use of a preparation according to any one of claims 1 to 28 for producing a preparation for treating acquired or inherited blood coagulation disorders.

31. The use of a preparation according to any one of claims 1 to 28 for producing a preparation for treating severe hemorrhages.

32. The use of a preparation according to any one of claims 1 to 28 for producing a preparation for the prophylaxis of hemorrhages, in particular in case of inherited blood coagulation disorders.

33. The use of a preparation according to any one of claims 1 to 28 for producing a preparation for substitution therapy.

34. The use of a preparation according to any one of claims 1 to 28 for producing a preparation for treating hemophilia B.

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